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A Specific and Sensitive Assay to Quantify the Herbicidal Activity of Chloroacetamides

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Abstract: Based on a recently discovered property of chloroacetamide herbicides—the inhibition of the incorporation of oleic acid into sporopollenin of *Scenedesmus acutus*—a rapid quantitative test was developed for chloroacetamide-type herbicidal activity. In this test, algal cells are incubated for 3 h with [^{14}C]oleic acid, saponified and the lipids (including non-saponifiable ones) extracted and discarded. The radioactivity incorporated into the residual non-lipid fraction is determined, and inhibition of this incorporation is used as a marker of chloroacetamide-type activity. Twenty-two agrochemical compounds were screened in this assay, which was found to be very sensitive, a 50% inhibition being reached with submicromolar herbicide concentrations. It is specific to chloroacetamides and related amides, since all these herbicides tested were potent inhibitors, while other herbicides were not. Highest inhibition was shown by cafenstrole followed by butachlor, fluthiamid, metazachlor, alachlor, dimethenamid, metolachlor and mefenacet. For these herbicides (with the exception of butachlor) sensitivity in the test was positively correlated ($r = 0{\cdot}984$) with their phytotoxic effect on the alga. © 1998 SCI

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Key words: chloroacetamides; amide herbicides; assay; oleic acid incorporation; *Scenedesmus acutus*; sporopollenin

1 INTRODUCTION

Forty years after the first chloroacetamide herbicide (allidochlor; CDAA) became available for agriculture[1] this class of biologically active substances still represents one of the most important agrochemicals for weed control. They are used as pre-emergence herbicides to reduce grass and some dicotyledonous weeds in oilseed rape, soybean, maize and other crops. Formulated with a safener, their use can be further extended by selectivity improvement. Despite their age and wide use, the primary site of action of this herbicide class is still unknown. Numerous physiological and biochemical effects have been reviewed,[2,3] including inhibition of lipid, protein and lignin synthesis, cell division, mineral uptake and increase of cell permeability. Since these reviews were written a reduction of acetate uptake[4] and of its incorporation into a DNA-associated protein[5] in the green alga, *Scenedesmus acutus*, has been reported. Recently, the possible inhibition of histone acetylation *in vitro* by these herbicides was ruled out.[6] In *S. acutus*, micromolar concentrations of alachlor or metazachlor were found to cause a sharp decrease in fatty acid desaturation.[7,8] The primary target site could not be convincingly ascribed to any of these effects, and it has been proposed that these herbicides could be multifunctional. However, lethal submicromolar concentrations of the herbicides suggest a single target site.[8,9]

Using *S. acutus* as a model organism, the most striking effect of chloroacetamides could be described: the inhibition of oleic acid incorporation into a non-lipid fraction (NLF) obtained after saponification of the algae. A 50% inhibition is achieved with 20–30 nM metazachlor after a very short incubation time.[8] The inhibition of incorporation of either C_1- or C_{10}-labelled

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oleic acid confirmed that oleic acid and not a short-chain metabolite is incorporated into the NLF.[8] This effect was not found in a metazachlor-tolerant *S. acutus* cell strain.[10] Apparently, this inhibition is specific to chloroacetamide or related herbicides, since it was also observed with alachlor, dimethenamid and mefenacet but not with diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] or oryzalin-4-dipropylamino-3,5-dinitrobenzenesulfonamide].[11] It was also related to herbicidally active chloroacetamides, since the non-herbicidal *R*-enantiomer of dimethenamid is inactive while the herbicidal *S*-enantiomer is very potent.[12] As shown by fractionation of the non-lipid fraction (NLF), inhibition of oleic acid incorporation into sporopollenin was responsible almost exclusively for the inhibition in the NLF.[13] The possible primary target for the chloroacetamides should be found within the metabolic route of oleic acid.

Several assays exist to characterize rapidly and quantitatively the mode of action of a phytotoxic chemical using relatively simple procedures. For example, the inhibition of oxygen evolution by photosynthesis inhibitors,[14] ethane formation by peroxidizing herbicides,[15] the inhibition of anthocyanin production in buckwheat by glyphosate[16] and the inhibition of amino acids with a simple *Lemna* test[17] are among those recently reviewed.[18] No such assay exists to determine the activity of chloroacetamides. The inhibition of oleic acid incorporation into the NLF by chloroacetamides is the most potent effect of these herbicides so far reported, and it occurs at very low concentrations within a short incubation time. In the present paper, this recently discovered property serves as the basis for a rapid, sensitive assay specific to herbicides with a chloroacetamide-type mode of action, that allows a quantitative determination of their phytotoxic activity.

2 MATERIALS AND METHODS

2.1 Plant material and culture conditions

Stock cultures of the unicellular green alga *Scenedesmus acutus* (no. 276-3a, Algae Collection, Göttingen, Germany) were grown in the light (80 μmol PAR m^{-2} s^{-1}) in cylindrical glass vessels (46 cm long, 4 cm diameter) containing 200 ml of liquid mineral medium that was continuously stirred by a flow of sterile air enriched with carbon dioxide (20 ml $litre^{-1}$) entering at the bottom of the vessels; the suspension was subcultured every other day as described elsewhere.[19] One day before the experiments, algae were subcultured into fresh medium starting with a cell density (pcv–packed cell volume) of 1 μl ml^{-1}. At the time of the experiments the pcv was approximately 2–3 μl ml^{-1}, corresponding to a chlorophyll (Chl) content of 15–20 μg ml^{-1}.

2.2 Assay procedure

One-day-old cultures of *S. acutus* were concentrated to 50 μg Chl ml^{-1}. The algal suspensions (1·8 ml per sample) were preincubated for 30 min with sodium hydrogen carbonate (1 M; 10 μl) and herbicide solution (2 μl; usually in EtOH) in capped 12-ml test tubes. The test tubes were placed almost horizontally on an orbital shaker in the light (55 μmol PAR s^{-1} cm^{-2}) at 25°C. Each assay set contained three negative controls without herbicide, positive controls with three concentrations (0·01, 0·1, or 1 μM) of metazachlor or another known chloroacetamide, and the same concentrations of the compounds (usually two at a time) to be tested. Except for control runs in triplicate all other samples were duplicated.

Herbicides (Fig. 1) were usually diluted in ethanol (lenacil in dimethyl sulfoxide) and added to the samples so that the concentration of the solvent was equal to or less than 1 ml $litre^{-1}$. The highest ethanol concentration of 1 ml $litre^{-1}$ had no effect on algal growth or incorporation of oleic acid into the NLF, and its concentration was the same in control and herbicide-treated algae. All herbicides were technical substances except for dichlormid (emulsifiable concentrate).

[1-^{14}C]Oleic acid (13 μl, *c.* 20 nmol; 2·04 TBq mol^{-1}, Amersham, Braunschweig, Germany) dissolved in toluene was placed into a silanized 12-ml glass tube and dried under nitrogen. Algal growth medium (5 ml) was added and the oleic acid dispersed by ultrasonication for 5 min. Once the algae had been preincubated for 30 min with herbicide, 200 μl of the algal growth medium containing oleic acid was added to the 1·8-ml algae culture and incubation proceeded further for another 3 h as above. The quantity of oleic acid given to the algae was estimated by directly counting the radioactivity (with the liquid scintillation cocktail, Ultima Gold, Packard, Frankfurt, Germany) in an aliquot of the medium supplied. The total amount of radioactivity per tube was approximately 1·7 kBq.

At the end of the 3-h incubation, incorporation of oleic acid into the algae was stopped by the addition of potassium hydroxide in methanol (100 g $litre^{-1}$; 7 ml) and the algae saponified for 30 min at 65°C. During this saponification a precipitate was formed. The sample was cooled, centrifuged (5 min, *c.* 1000*g*) and the supernatant discarded. The pellet, which represented the NLF, was washed once with chloroform + methanol (2 + 1 by volume; 4 ml). The resulting fraction was suspended in methanol (1 ml) and counted for radioactivity. This fraction is equivalent to the 'saponification precipitate' described earlier by Couderchet and Böger.[8]

2.3 Growth inhibition measurements

Scenedesmus acutus cultures (120 ml) were grown in cylindrical glass vessels as in Section 2.1. At the begin-

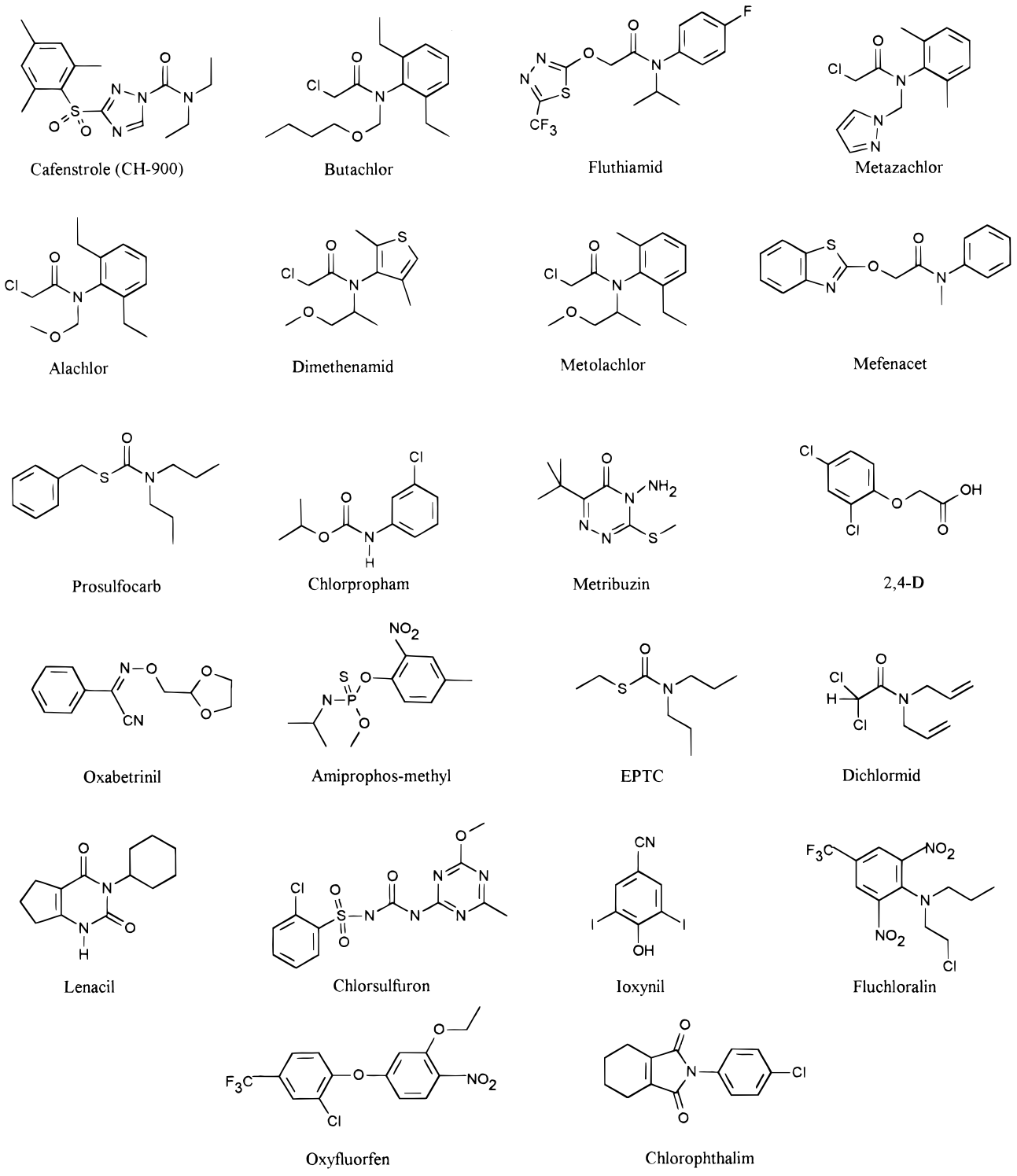

Fig. 1. Structure of the compounds tested for their inhibition of oleic acid incorporation into a nonlipid fraction of *Scenedesmus acutus.*

ning of the experiments the cell density (pcv) of the cultures was 1–2 μl ml^{-1}, corresponding to a Chl content of 7–15 μg ml^{-1}. Herbicide (0·01 M in ethanol; 60 μl) was added to the culture. After 50 h of incubation, pcv and Chl were determined and compared to those of control cultures that had not been exposed to any herbicide.

2.4 Statistics

An experiment was considered valid when an inhibition could be observed with the positive chloroacetamide controls. The experiments were repeated at least twice. Results shown here are mean (±standard error) of all experiments conducted for each compound.

3 RESULTS AND DISCUSSION

In the absence of herbicide, incorporation of radioactivity into the NLF was between 50 and 65 Bq. At herbicide concentrations as low as 0·01 μM some of the compounds tested inhibited this incorporation, while others had no effect or even stimulated the incorporation (Table 1). After incubation with 1 μM herbicide, inhibition was highest (73%) with cafenstrole, a *N*,*N*-disubstituted amide with strong herbicidal activity, particularly against *Echinochloa* sp.[20] An inhibition of 50% was reached at less than 1 μM for butachlor, fluthiamid, metazachlor, alachlor, dimethenamid and metolachlor (Table 1). Inhibition with metazachlor was comparable to that found in different incubation conditions, with 50% inhibition reached between 0·01 and 0·1 μM.[8] The results obtained under these conditions are in total agreement with those obtained earlier, in which the inhibition was stronger with metazachlor than it was with alachlor, dimethenamid and finally mefenacet.[11] Dimethenamid and metolachlor exist as two stereoisomers, the *S* isomer being herbicidally active while the *R* form is not.[12,21] The relatively low inhibition of oleic acid incorporation into the NLF found for these two herbicides is that of the racemates. Inhibition by the *S* form of dimethenamid[12] or metolachlor (unpublished results) was markedly higher. Inhibition by 1 μM mefenacet was 41% lower than with the other acetamide herbicides, which corroborates the results obtained before in which mefenacet was found to be more than ten times less efficient than metazachlor.[11] Mefenacet is an oxyacetamide herbicide, a class related to chloroacetamides and having many characteristics in common.[22,23] Its effect on the incorporation of oleic acid into the NLF is additional evidence that chloroacetamides and oxyacetamides may share a primary target. The other oxyacetamide tested, fluthiamid, confirms the inhibitory activity of this class of compounds in the assay. As in the assay, fluthiamid is also much more active as a herbicide than is mefenacet.[24]

For this first group of herbicides, inhibition was concentration-dependent, indicating a specific action of the compound. In contrast, 50% inhibition was not reached with the other herbicides tested. Some of them inhibited more than 20% but, for the majority, inhibition was not significant. Furthermore, even though some compounds, such as chlorpropham, could significantly inhibit incorporation [26(±4%)] of oleic acid,

TABLE 1
Inhibition of [^{14}C]Oleic Acid Incorporation into the Non-lipid Fraction (NLF) of *Scenedesmus acutus* by Herbicides of Various classes and Safeners

Compound tested	*Inhibition* (%) (±*SD*)[a]		
	0·01 μM	*0·1 μM*	*1 μM*
Cafenstrole (CH 900)	30 (±14)	53 (±2)	73 (±3)
Butachlor	26 (±10)	50 (±4)	70 (±6)
Fluthiamid	32 (±5)	46 (±15)	65 (±5)
Metazachlor	17 (±13)	51 (±12)	64 (±3)
Alachlor	29 (±7)	49 (±14)	62 (±5)
Dimethenamid	17 (±11)	41 (±10)	53 (±3)
Metolachlor	28 (±1)	41 (±4)	50 (±5)
Mefenacet	3 (±2)	24 (±10)	41 (±3)
Prosulfocarb	17 (±9)	24 (±1)	26 (±8)
Chlorpropham	23 (±10)	18 (±1)	26 (±4)
Metribuzin	3 (±3)	4 (±14)	22 (±8)
2,4-D	2 (±3)	−7 (±3)	15 (±1)
Oxabetrinil	26 (±13)	20 (±14)	13 (±8)
Amiprophos-methyl	16 (±17)	12 (±12)	12 (±8)
EPTC	−10 (±1)	−7 (±2)	8 (±3)
Dichlormid	−4 (±19)	5 (±3)	7 (±2)
Lenacil	−4 (±11)	−3 (±15)	6 (±3)
Chlorsulfuron	−7 (±1)	−1 (±3)	3 (±7)
Ioxynil	−8 (±1)	3 (±2)	0 (±9)
Fluchloralin	1 (±1)	−4 (±0)	−2 (±1)
Oxyfluorfen	−3 (±9)	−34 (±1)	−52 (±12)
Chlorophthalim	−4 (±10)	−24 (±6)	−95 (±34)
Oxyfluorfen[b]			6 (±20)
Chlorophthalim[b]			8 (±17)

[a] Two to eight independent determinations.
[b] Incubated in the dark.

this did not seem to be specific, since the inhibition was not concentration-dependent.

Oxyfluorfen and chlorophthalim increased radioactivity incorporation into the NLF, and this effect depended on the concentration of the two herbicides. These herbicides inhibit protoporphyrinogen oxidase in the synthesis of chlorophyll and belong to the peroxidizing herbicides, which induce the formation of highly reactive oxygen species that can peroxidize lipids, thereby destroying cell membranes.[25] Their herbicidal effect on plants is only exhibited in the light. Similarly, when the algae treated with the two peroxidizers were protected from light, even the highest concentration (1 μM) tested did not influence oleic acid incorporation into the NLF (Table 1). Thus, as suggested before,[13] it could be speculated that incorporation of oleic acid into the NLF, particularly into sporopollenin, may be stimulated by some oxidative reactions in the cell and that these or one of the subsequent reactions could be inhibited in some way by chloroacetamide herbicides.

Inhibition of *Scenedesmus* growth was investigated with those herbicides that showed more than 30% inhibition of the incorporation of oleic acid into the NLF. Whether pcv or Chl was used to quantify growth, all these eight herbicides were effective (Table 2). Except for butachlor, growth inhibition was positively correlated ($r = 0{\cdot}984$ or $r = 0{\cdot}980$) with inhibition of oleic acid incorporation (Fig. 2). It was highest with cafenstrole, followed by fluthiamid and metazachlor, and lowest with metolachlor and mefenacet. Detoxification by the alga may be faster for butachlor than for the other herbicides, leading to a pronounced inhibition of oleic acid incorporation after 3 h, while inhibition of growth after a 50-h incubation is only moderate. Detoxification of chloroacetamides, at least metazachlor, by the alga also occurs by glutathione conjugation (Couderchet and Böger, unpublished results) and glutathione-*S*-transferase activity has been found in *S. acutus*.[26] However, nothing is known about different activities against different chloroacetamides. At the moment, the outlier position of butachlor cannot be explained.

TABLE 2
Inhibition of the Growth of *Scenedesmus acutus* Suspension Cell Culture by Various Herbicides

	Growth inhibition (%) (±*SD*)[a]	
Compound tested (5 μM)	*pcv*	*Chl*
Cafenstrole (CH 900)	54 (±10)	84 (±6)
Butachlor	27 (±4)	48 (±3)
Fluthiamid	47 (±1)	78 (±6)
Metazachlor	43 (±3)	73 (±7)
Alachlor	38 (±8)	73 (±8)
Dimethenamid	23 (±9)	60 (±18)
Metolachlor	15 (±15)	51 (±19)
Mefenacet	11 (±12)	35 (±14)
Oxyfluorfen	100 (±0)[a]	100 (±0)[a]
Chlorophthalim	100 (±0)[a]	100 (±0)[a]

a Three to six independent determinations.
[b] The cells were totally bleached.

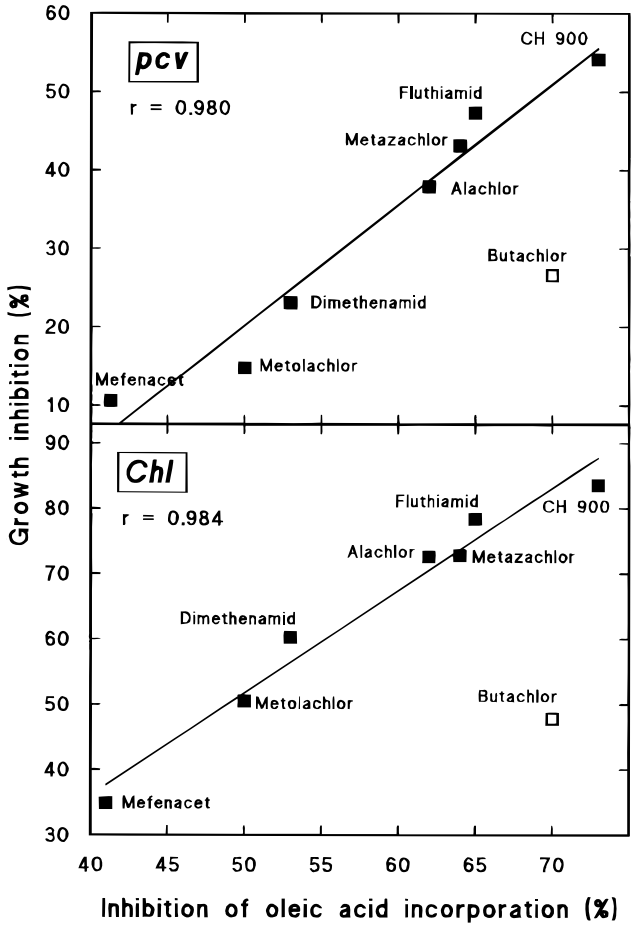

Fig. 2. Correlation between inhibition of oleic acid incorporation into a nonlipid fraction of *Scenedesmus acutus* and growth inhibition of a culture of *S. acutus* by some herbicides. Packed cell volume (pcv) and chlorophyll content of the culture (Chl) were used as growth parameters. Herbicide concentration was 1 μM in the oleic acid assay and 5 μM for growth inhibitions.

Inhibition of growth by oxyfluorfen and chlorophthalim was complete (Table 2) but these compounds did not inhibit oleic acid incorporation into the NLF, indicating that reduction of oleic acid incorporation is not simply due to decreased growth, but that this effect seems specific for herbicides with a chloroacetamide-like mode of action.

Two of the herbicides (cafenstrole and fluthiamid) that displayed the highest inhibition of oleic acid incorporation are not chloroacetamides. However, these two herbicides (see also mefenacet) like the chloroacetamides include a *N*,*N*-disubstituted amide function. It could be speculated that this function is necessary for the inhibitory effect, but it is not the only requirement, since the *R* isomer of dimethenamid and BAS 128682 [2-methoxy-*N*-(2,6-dimethylphenyl)-*N*-(1*H*-pyrazol-1-

ylmethyl)acetamide] are also *N*,*N*-disubstituted but do not inhibit oleic acid incorporation nor growth of *Scenedesmus*.[11,12]

4 CONCLUSIONS

Previously, inhibition of oleic acid incorporation into the non-lipid fraction was obtained after incubating the cells in larger volumes and in continuously aerated medium.[8,11–13] In this investigation, inhibition occurred in tightly capped test tubes and in smaller volumes making the extraction more practical and thereby faster and more economical. With two negative controls, two positive controls, and two replicates per substance, it is possible for one person routinely to test 10 substances per day.

Thus, with the conditions described here, inhibition of oleic acid incorporation into the NLF of *Scenedesmus* provides (to our knowledge) the first and only rapid, sensitive and specific test for chloroacetamide-like activity. It should be noted that the NLF includes some fractions whose oleate incorporation was not sensitive to herbicides. Accordingly, the inhibition described here never attained 100%. This could be achieved, however, by using a residue obtained by acid acetolysis of the NLF.[13] Doing so, the assay gains reliability but becomes laborious and requires more time.

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